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THE EFFECT OF CERTAIN CHLORIDES SINGLY AND COMBINED IN PAIRS ON THE ACTIVITY OF MALT DIASTASE[1]

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The effects produced by single inorganic salts in aqueous solution upon an organism and the modification of these effects by the presence of other salts has been the subject of considerable research. It has frequently been found that a definitely toxic influence of one salt may be completely removed by the addition of a second salt, itself also markedly toxic when used alone. This mutual counteraction by salts of the toxic influence of each other has been termed antagonism. While single salts and their combinations do not, of course, affect all organisms in the same way, yet different investigators have shown antagonistic salt action in the case of many organisms and with many chemical compounds. Thus, LOEW[2] demonstrated that the injurious action of certain concentrations of magnesium nitrate upon *Spirogyra majuscula* is inhibited by the presence in the medium of a proper amount of calcium nitrate. Similarly LOEB[3] found an antagonism to exist between the nitrates of calcium and of sodium in their influence upon the development of the eggs of *Fundulus;* and OSTERHOUT,[4] working with plants, has shown the same kind of antagonism between the salts of magnesium and of potassium. That calcium and potassium influence each other in their effect upon certain bacteria has been brought out by LIPMAN.[5]

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[1] Botanical contribution from the Johns Hopkins University. No. 23.

[2] LOEW, O., Über die physiologischen Functionen der Calcium- und Magnesium-Salze im Pflanzenorganismus. Flora **75**:368–394. 1892.

[3] LOEB, J., Studies on the physiological effects of the valency and possibly the electrical charges of ions. I. The toxic and antitoxic effects of ions as a function of their valency and possibly their electrical charge. Am. Jour. Physiol. **6**:411–433. 1902.

In a theoretical consideration of the possible causes of chemical stimulation, it is suggested that, since many vital phenomena seem to be dependent upon enzymes, it is possible that the influence of chemical substances upon the activity of plants and animals may be due in some measure to the action of such substances in accelerating or retarding enzyme activity. With these ideas in mind, it was undertaken to study the relation between the catalytic action of some enzyme and the concentration in the medium of certain salts, to determine, if possible, whether enzyme catalysis is influenced independently by two salts simultaneously present in the medium, or whether either salt increases or decreases the accelerating or retarding influence of the other upon such catalytic reactions.

The studies here reported bear upon this question and were carried out in part in the new Laboratory of Plant Physiology of the Johns Hopkins University, at Homewood, and in part in the Biological Laboratory of the Johns Hopkins University. The writer is greatly indebted to Professor BURTON E. LIVINGSTON, under whom this investigation was conducted, for his many helpful suggestions and criticisms.

Materials

The enzyme chosen for investigation was, for practical reasons, malt diastase (Merck's "diastase of malt absolute"), and the salts used were chlorides of sodium, potassium, calcium, magnesium, copper, and iron (Kahlbaum's "chemically pure" salts were exclusively used). The activity of the diastase was measured in terms of the length of time required for it to render a starch paste (of washed maize starch) powerless to give color reactions with iodine.

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[4] OSTERHOUT, W. J. V., The antagonistic action of magnesium and potassium. BOT. GAZ. **45**:117–124. 1908.

[5] LIPMAN, CHARLES B., Toxic and antagonistic effects of salts as related to ammonification by *Bacillus subtilis*. BOT. GAZ. **48**:105–125. 1909.

All water used was distilled from glass and condensed in a well seasoned glass condenser. The various pieces of apparatus coming in contact with the solutions or mixtures of the experiments were carefully cleansed in this water before use.

Method

The method here employed for determining the duration of hydrolysis is similar to that described by SHERMAN, KENDALL, and CLARK as the liquefaction method for estimating diastatic activity.[6] The exact procedure in the present study was as follows: A sufficient quantity of starch for the entire study was purchased at one time and thoroughly mixed. Of this material 2.5 grams were weighed out, placed in a porcelain dish, and by means of a glass rod rubbed into a suspension with a little water. This mixture was then diluted to a volume of 500 cc., boiled two minutes, and then strained through cheesecloth, thus making a 0.5 per cent starch paste, a homogenous liquid which flows freely and is easily measured from a burette.

With the exception of a small amount used in preliminary tests, all the diastase for the investigation was purchased at one time and thoroughly mixed to insure uniformity. It was preserved in a glass-stoppered bottle and showed no signs of deterioration during the several months the study was in progress. A 2 per cent solution of this diastase was prepared in much the same manner as that employed in making the starch paste. Two grams of the dry material were first thoroughly suspended in a small amount of water and then diluted to a volume of 100 cc., after which the solution was filtered. Fresh diastase solution and also fresh starch paste were prepared at the beginning of each experiment, to preclude the possibility of error resulting from the growth of microorganisms. No antiseptic was used in the various mixtures.

Stock salt solutions, sufficient in quantity for the entire study, were prepared at the beginning of the investigation, in concentrations of 1–4 gram molecules per liter of solution, and from these the requisite dilutions were made. The iodine solution used in the tests was originally prepared in quantity sufficient for the whole research in a concentration of 1.5 grams of iodine and 3 grams of potassium iodide per liter of water.

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[6] SHERMAN, H. C., KENDALL, E. C., and CLARK, E. D., Studies on amylases. I. An examination of methods for the determination of diastatic power. Jour. Am. Chem. Soc. **32**: 1073–1087. 1910; references to the earlier literature are given here.

In experimentation the various mixtures were placed in large test tubes (30 mm. in diameter and 150 mm. deep), which were suspended through the suitably perforated top of a cylindrical water-bath. The water of the bath stood always at a higher level than that of the liquid in the tubes, which were some distance apart, the outer ones being several centimeters from the walls of the bath. It was thus assured that the mixtures in the tubes were maintained at the same temperature as the surrounding water. The temperature of the bath was kept nearly constant at 50° C., by means of an ordinary mercury regulator and a gas flame below. The variations in temperature were between the limits of 48° and 52°. Since the computations of the velocity of diastatic activity were always made in terms of the velocity of the reaction in controls within the same series, the possible effects of temperature fluctuations should be exceedingly slight.

A series of mixtures was usually prepared in the following manner: In each of a series of tubes was placed the proper amount of salt solution at a concentration in each case of four times[7] the required concentration for each particular tube. To this were added 5 cc. of water, in the cases where the action of a single salt was to be tested, or an equal quantity of the solution of the other salt, in those where salt combinations were to be used; 8 cc. of starch paste (0.5 per cent) were then added to each tube, followed by 2 cc. of the previously prepared diastase solution. The tubes, containing 20 cc. of mixture, were immediately placed in the water-bath. It is thus apparent that all test mixtures contained the same quantity (0.2 per cent or 0.004 gm.) of the starch and of the diastase.

An experiment was usually made up of (1) a series of 7 different concentrations of some salt used singly; (2) a series of 7 different concentrations of another salt used singly, and (3) a series, usually in duplicate, of the 7 concentrations of the two salts in binary combinations. These with the two control tubes made a total of

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[7] For 2m mixtures 10 cc. of the 4m stock salt solution were placed in each tube; this when diluted to 20 cc. by the addition of the starch and diastase gave the required concentration of the salt.

30 tubes to be tested at one time. The control tubes were prepared in exactly the same way as the others, except that water was substituted for the salt solution. In order to determine whether or not the salt solutions alone had any appreciable hydrolytic effect upon the starch, tubes were frequently made up with the salt solution and starch content the same as those in the experiment but without the diastase. These were placed in the water-bath together with the other tubes and tested from time to time in the usual manner, but in no case was there any variation from the typical blue color of the starch-iodine reaction. It was thus clear, as was to be expected, that while the salts could influence the velocity of the action of the diastase on starch, they were themselves apparently incapable of any appreciable hydrolytic action.

To determine whether the contents of any given tube had reached the end point, a single drop of the stock iodine solution was placed in each of two small vials (10 mm. in diameter and 70 mm. high). To one vial was added 1 cc. of water and to the other a like quantity of the mixture from the proper tube, carefully pipetted out. The two vials were then observed against a white background in diffused light, and if their contents appeared to be of the same color (a pale yellow) the experimental end point was considered to have been attained. It has been found that by this method of determining the end point, the color produced in a starch paste containing but 0.0002 of 1 per cent can be readily detected. This sensitiveness lies well within the limits of error of the other operations of the experiments. Final failure of one of the starch-diastase mixtures to produce color (blue, purple, red, brown, etc.) does not necessarily mean that all of the original starch content has been converted into a reducing sugar, but merely that all of the starch has been so altered as to be no longer able to form colored compounds with iodine—showing that one or several of the steps of the process of hydrolysis have been completed. In this connection it may be remarked that LANG[8] has shown with pancreatic amylase that the amount of a reducing sugar present at the time when the starch paste first fails to color with iodine does not represent and is not proportional to the original amount of starch. The present investigation is therefore not directly comparable with such investigations as those of KJELDAHL,[9] KELLERMAN,[10] MCGUIGAN,[11] and others, who measured diastatic activity by determining the amount of a reducing sugar formed in a given time. For a more complete bibliography on the effects of salts on diastatic action, the reader is referred to EFFRONT,[12] GREEN,[13] OPPENHEIMER,[14] EULER,[15] as well as to the literature cited here.

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[8] LANG, S., Über die Einwirkungen der Pankreasdiastase auf Stärkearten verschiedenen Herkunft. Zeitschr. Exper. Path. u. Therapie **8**:279–307. 1910.

Experimentation

The water, diastase, and starch used in this work were tested for neutrality in the course of the investigation, and it was found that while the water and starch-paste were neutral to phenolphthalein, the diastase solution was strongly acid, requiring 1.2 cc. of 0.01 normal hydrate to neutralize 2 cc. of the stock solution. Thus each tube of mixture as actually used possessed an acidity equivalent to 1/1660, or 0.000602 normal. Several series were carried out by neutralizing or partially neutralizing (with sodium hydrate) this acidity of the diastase, and a strong retardation was evident when even a small quantity of the alkali was added. Thus the use of 5 cc. of 0.0005 alkali diluted to 20 cc. by the addition of the usual amounts of diastase solution, starch-paste, and water (reducing the acidity of the mixture to 0.000477 normal) caused a retardation of 13 per cent as compared with the control without the alkali. With a further decrease in the acidity to 0.000352 normal, there was apparently but little action of the diastase, and when the original acidity was completely neutralized, no alteration in the starch could be detected by the iodine test, after a period of 5 hours in the water-bath.

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[9] KJELDAHL, Recherches sur les ferments producteurs de sucre. Compt. Rend. Lab. Carlsberg **1**:109–157. 1879.

[10] KELLERMAN, KARL F., The effects of various chemical agents upon the starch-converting power of taka diastase. Bull. Torr. Bot. Club **30**:56–70. 1903.

[11] MCGUIGAN, H., The relation between the decomposition-tension of salts and their antifermentative properties. Am. Jour. Physiol. **10**:444–451. 1904.

[12] EFFRONT, JEAN, Enzymes and their applications. (PRESCOTT'S trans.) New York. 1902.

[13] GREEN, J. REYNOLDS, The soluble ferments and fermentation. Cambridge University Press. 1901.

[14] OPPENHEIMER, KARL, Die Fermente und ihre Wirkungen. Leipzig. 1910.

[15] EULER, HANS, Allgemeine Chemie der Enzyme. Wiesbaden. 1910.

A determination of the resistances of the starch and diastase solutions by means of a Wheatstone bridge and the calculation of corresponding conductivities showed the presence of a small quantity of electrolytes in the mixture. These must have modified to some extent the effect of the addition of the different salts in the experiments. Since, however, the same concentrations of starch and diastase were employed throughout the investigation, the electrolytes originally present are to be considered as constant in quantity. Furthermore, the concentrations of the salts used were usually comparatively high, so that any resulting errors due to what may be termed electrolytes of impurity (or of constitution) of the mixture used must be relatively slight.

It was observed in many cases that, in mixtures containing salts at the higher concentrations used, a flocculent precipitate was gradually formed during the experiment. This precipitate was isolated and gave no starch reaction with iodine. No relation could be detected between its occurrence or amount and the diastatic activity. It seems, therefore, that the effect of the presence of a salt in increasing or decreasing diastatic activity cannot be due to a salting out from the solution of either diastase or starch. This is in accord with the conclusion reached by COLE[16] in his work on ptyalin, but contrary to what might seem to be the case from the investigations of HARDY.[17] In this connection it is interesting to note that MUNTER[18] has recently shown that many of the salts of the alkalies and alkaline earths are ineffective for the precipitation of the diastase of *Aspergillus oryzae*.

Testing was begun when the mixtures had remained in the water-bath one hour, and was continued thereafter at intervals of 15 minutes until the conclusion of the experiment. Thirty or more similar vials were prepared before each test, each with its drop of iodine solution, and the samples were consecutively pipetted into these from the tubes, following the order in which the diastase had been originally added. The pipette was thoroughly washed after each use and thoroughly clean vials were always employed.

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[16] COLE, S. W., Contributions to our knowledge of the action of enzymes. Part I. The influence of electrolytes on the action of amylolytic ferments. Jour. Physiol. **30**: 202–220. 1903.

[17] HARDY, W. B., A preliminary investigation of the conditions which determine the stability of irreversible hydrosols. Proc. Roy. Soc. London **66**: 110–125. 1900.

[18] MUNTER, F., Über Enzyme. Landwirtsch. Jahrb. Erganzband III. **39**: 298–314. 1910.

The number of minutes which elapsed before the end point was reached for each mixture was recorded, thus furnishing a means for the quantitative comparison of the enzyme activity under the different conditions. For example, a series of 2, 1/2, 1/8, 1/32, 1/128, 1/512, and 1/2048 molecular sodium chloride gave for the respective time periods 165, 165, 150, 180, 315, 285, and 300 minutes, the control mixture (without any added salt) reaching the end point in 285 minutes.[19] The intensity of the enzyme action in experiments such as these is, of course, for any given mixture reciprocally proportional to the number of minutes required for that mixture to reach the end point. Thus the ratio of the time period of the control to that of any mixture is obviously a measure of the velocity of the hydrolysis for that mixture in terms of the velocity of the control. Thus, the relative velocities or intensities of starch hydrolysis for the experiment just cited become, respectively, 285/165,

TABLE I

EFFICIENCY OF DIASTATIC ACTION AS AFFECTED BY VARIOUS CONCENTRATIONS OF SODIUM AND POTASSIUM CHLORIDES, SINGLY AND COMBINED

SINGLE SALTS				COMBINATIONS (Molecular proportions 1:1)	
NaCl		KCl			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
2m	1.51	2m	1.77	4m	....
m/2	1.71	m/2	2.19	Mol.	1.38
m/8	1.82	m/8	1.61	m/4	1.93
m/32	1.42	m/32	1.39	m/16	1.92
m/128	0.85	m/128	0.85	m/64	1.07
m/512	0.95	m/512	0.93	m/256	0.89
m/2048	1.02	m/2048	1.00	m/1024	0.96

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[19] The time periods for the two control tubes were the same in all series except three, and in these cases the two results (differing but slightly) have been averaged.

285/165, 285/150, 285/180, 285/315, 285/285, and 285/300, or 1.73, 1.73, 1.90, 1.58, 0.90, 1.00, and 0.95, when the velocity of the reaction without added salt is taken as unity.

TABLE II

EFFICIENCY OF DIASTATIC ACTION AS AFFECTED BY VARIOUS CONCENTRATIONS OF SODIUM AND CALCIUM CHLORIDES, SINGLY AND COMBINED

SINGLE SALTS				COMBINATIONS (Molecular proportions, NaCl 2 : $CaCl_2$ 1)	
NaCl		$CaCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
2m	1.51	Mol.	Retardation	3m	Retardation
m/2	1.71	m/4	1.33	3m/4	1.21
m/8	1.82	m/16	2.89	3m/16	3.29
m/32	1.42	m/64	3.69	3m/64	3.40
m/128	0.85	m/256	3.01	3m/256	2.75
m/512	0.95	m/1024	1.88	3m/1024	1.85
m/2048	1.03	m/4096	1.07	3m/4096	1.17

TABLE III

EFFICIENCY OF DIASTATIC ACTION AS AFFECTED BY VARIOUS CONCENTRATIONS OF SODIUM AND MAGNESIUM CHLORIDES, SINGLY AND COMBINED

SINGLE SALTS				COMBINATIONS (Molecular proportions, NaCl 4 : $MgCl_2$ 1)	
NaCl		$MgCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
2m	1.51	m/2	Retardation	5m/2	Retardation
m/2	1.71	m/8	2.25	5m/8	0.98
m/8	1.82	m/32	2.07	5m/32	1.90
m/32	1.42	m/128	1.97	5m/128	2.21
m/128	0.85	m/512	1.35	5m/512	1.54
m/512	0.95	m/2048	1.07	5m/2048	0.89
m/2048	1.02	m/8192	1.00	5m/8192	0.99

These numbers may be termed the ratios of the enzyme activity or of efficiency. Such a computation as the foregoing has been made in each case, always with the enzyme activity of the control for the particular experiment in question taken as unity, and it is with the efficiency ratios for the diastase in the presence of the various concentrations of the different salts that this paper has to deal. These ratios should be comparable throughout the entire study; where they are less than unity the salt or salts have exerted a retarding influence upon hydrolysis; where they equal unity the salt treatment has been without effect; and where they are greater than unity the effect of the salts has been to accelerate the

TABLE IV

EFFICIENCY OF DIASTATIC ACTION AS AFFECTED BY VARIOUS CONCENTRATIONS OF POTASSIUM AND CALCIUM CHLORIDES, SINGLY AND COMBINED

Single salts				Combinations (Molecular proportions, KCl 2:$CaCl_2$ 1)	
KCl		$CaCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
2m	1.77	Mol.	Retardation	3m	Retardation
m/2	2.19	m/4	1.33	3m/4	1.24
m/8	1.61	m/16	2.89	3m/16	2.73
m/32	1.39	m/64	3.69	3m/64	2.87
m/128	0.85	m/256	3.01	3m/256	2.87
m/512	0.93	m/1024	1.88	3m/1024	2.13
m/2048	1.00	m/4096	1.07	3m/4096	1.14

TABLE V

EFFICIENCY OF DIASTATIC ACTION AS AFFECTED BY VARIOUS CONCENTRATIONS OF POTASSIUM AND MAGNESIUM CHLORIDES, SINGLY AND COMBINED

Single salts				Combinations (Molecular proportions, KCl 4:$MgCl_2$ 1)	
KCl		$MgCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
2m	1.77	m/2	Retardation	5m/2	Retardation
m/2	2.19	m/8	2.25	5m/8	1.07
m/8	1.61	m/32	2.07	5m/32	1.83
m/32	1.39	m/128	1.97	5m/128	2.07
m/128	0.85	m/512	1.35	5m/512	1.20
m/512	0.93	m/2048	1.07	5m/2048	1.12
m/2048	1.00	m/8192	1.00	5m/8192	1.08

action of the enzyme. Of course the combination treatments possess a total salt concentration equal to the sum of the two individual concentrations of the single salts which have been combined.

TABLE VI

Efficiency of diastatic action as affected by various concentrations of magnesium and calcium chlorides, singly and combined

Single salts				Combinations (Molecular proportions, $MgCl_2$ 1 : $CaCl_2$ 2)	
$MgCl_2$		$CaCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
m/2	....	Mol.	Retardation	3m/2	Retardation
m/8	2.25	m/4	1.33	3m/8	1.06
m/32	2.07	m/16	2.89	3m/32	3.06
m/128	1.97	m/64	3.69	3m/128	3.61
m/512	1.35	m/256	3.01	3m/512	2.65
m/2048	1.07	m/1024	1.88	3m/2048	2.09
m/8192	1.00	m/4096	1.07	3m/8192	1.16

TABLE VII

Efficiency of diastatic action as affected by various concentrations of ferric and cupric chlorides, singly and combined

Single salts				Combinations (Molecular proportions, 1 : 1)	
Fe_2Cl_6		$CuCl_2$			
Concentration in mixture	Efficiency	Concentration in mixture	Efficiency	Total salt concentration in mixture	Efficiency
m/1024	Retardation	m/1024	Retardation	m/512	Retardation
m/2048	1.45	m/2048	3.50	m/1024	Retardation
m/4096	3.55	m/4096	2.62	m/2048	3.17
m/8192	3.91	m/8192	1.26	m/4096	3.59
m/16,384	1.98	m/16,384	1.00	m/8192	1.92
m/32,768	1.57	m/32,768	1.00	m/16,384	1.45
m/65,536	1.00	m/65,536	1.00	m/32,768	1.00

The total salt concentrations for the several combinations are given in the tables. It was impracticable to combine 2m NaCl with 2m KCl, so this combination was not tested. The molecular proportions at which any two salts were combined are clear from the concentrations of the single salts standing in the same line with the datum for the combination, but these proportions are stated at the head of the columns of combinations. In those cases where the word "retardation" occurs in the efficiency column, this denotes that the efficiency here is much below that of the control without added salt, the end point not having been reached at the close of the experiment in question.

EFFECT OF SINGLE SALTS

From the foregoing tables it is apparent that all the salts used in this investigation, at some concentrations (both singly and in combination), increase the rate of hydrolysis over that of the control without added salt; at certain other concentrations they retard this process; and at still others they have apparently no influence on diastatic activity as measured in this study. Thus, with the chlorides of sodium and potassium, used singly, it may be seen that with a concentration of m/2048 the effect is practically the same as in the control with distilled water. An increase in salt content, however (to m/512), results in a slight retardation of diastatic action; while with a further increase of added salt (to m/128), the point of maximum retardation, as found in this investigation, is attained. All higher concentrations used, of these two salts, accelerate hydrolysis, the points of maximum acceleration being at a concentration of m/8 for sodium chloride and of m/2 for the potassium salt. Possibly the most striking feature of the results shown in table I is that retardation apparently occurs only at relatively low concentrations. To supplement the evidence obtained from the experiments, that there was a retardation with m/128 sodium chloride and potassium chloride as here employed, five additional series were carried out with the two salts singly at this concentration accompanied by the required controls without added salt, all made up and tested in the usual manner. In every case a marked retardation was evident in the mixtures containing the salts, as compared with the controls. This retardation cannot be due to a partial neutralization of the acidity of the diastase by small amounts of free alkali present in the salt solutions (the latter were very slightly alkaline to phenolphthalein), for table I shows that still higher concentrations of these two salt solutions, containing proportionately more alkali, unquestionably accelerate diastatic activity.

So far as the writer has been able to learn, such retardation with dilute concentrations of sodium and potassium chlorides has not been recorded heretofore. KUEBEL,[20] working with saliva, showed a slight decrease in the acceleration of hydrolysis at low concentrations of these two salts, but found no retardation such as is shown in table I, m/128 being the most dilute solution he used. This writer found a maximum acceleration between m/32 and m/128 for these two salts, and a retardation with high concentrations. He used a colorimetric method for determining the velocity of hydrolysis, comparing with tubes of colored liquids the various colors produced by the addition of iodine after a given time period. It is possible that results thus obtained may not be comparable with those of the present investigation, as the time intervals of transition between the different colored dextrins and between the last colored product formed, and achroodextrin may not be in the same proportion when the enzyme is activated by a salt as when no salt is added. Of course it must also be remembered that KUEBEL'S enzymatic mixture was saliva, while that here studied was barley diastase.

GRUTZNER,[21] using the same method of estimating diastatic activity as did KUEBEL, but working with pancreatic amylase, found the optimum concentration of sodium chloride to be between m/8 and m/32.

For dialyzed ptyalin COLE found a slight decrease in the acceleration with m/3000 sodium chloride, though acceleration at this point was still considerable. He found the point of maximum acceleration to lie between m/4 and m/300. Experiments have been carried out upon the effect of sodium chloride on diastatic action by other investigators, with varying results.

Calcium and magnesium chlorides, used singly, affect hydrolysis somewhat differently from the two monovalent salts. A decided retardation is evident at high concentrations, molecular for the calcium salt and m/2 for that of magnesium. This is in marked contrast with the strong acceleration evident for sodium and potassium chlorides even at much higher concentrations. The points of maximum acceleration for calcium and magnesium chlorides, as here determined, occur at a concentration of m/64 for the former and of m/8 for the latter. From these points the acceleration falls with the gradual decrease in salt content to m/4096 calcium chloride, which accelerates but slightly, and to m/8192 magnesium chloride, which is apparently without influence. No retardation at low concentrations, as in the case of the monovalent salts, is here apparent.

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[20] KUEBEL, F., Über die Einwirkung verschiedener chemischer Stoffe auf die Thätigkeit des Mundspeichels. Archiv für die gesammte Physiologie **76**:276–305. 1899.

[21] GRUTZNER, P., Über die Einwirkung verschiedener chemischer Stoffe auf die Thätigkeit des diastatischen Pankreasfermentes. Archiv für die gesammte Physiologie **91**:195–207. 1902.

The effect of the salts of the heavy metals on diastatic action (with the possible exception of mercury) have not received as much attention as those of the alkalies or the alkaline earths, which are so uniformly present in the environment of most organisms. From table VII it is apparent that the chlorides of iron and copper, as used in this investigation, retard diastatic action markedly even when present in very small quantities. Acceleration occurs in very dilute solutions, as compared with the accelerating concentrations of the other salts, the region of acceleration being found to lie between concentrations of m/2048 and m/8192 for cupric chloride, and between those of m/2048 and m/32,768 for ferric chloride, with maximum accelerations at m/2048 and m/8192, respectively. WOHLGEMUTH,[22] working with salivary diastase, found that colloidal solutions of these metals in comparatively dilute concentrations retarded diastatic action. He did not find the strong acceleration which is so apparent in the present investigation, possibly because of the fact that his metals were in colloidal condition, or because of the few concentrations that he employed. According to the data here presented, calcium chloride appears to be exceptionally effective in accelerating diastatic action, which is in accord with the findings of LISBONNE,[23] working upon the effect of this salt in restoring the activity of dialyzed salivary and pancreatic amylases in the presence of demineralized starch.

[22] WOHLGEMUTH, J., Untersuchungen über Diastasen. Biochem. Zeitschr. **9**:10–42. 1908.

[23] LISBONNE, MARCEL, Influence des chlorures et de phosphates sur la saccharification de l'amidon déminéralisé par les amylases salivaire et pancréatique. Compt. Rend. Soc. Biol. **70**:207–209. 1911.

In the present study with ferric chloride, at a concentration of m/4096 an acceleration was found slightly higher than any obtained with the various concentrations of copper and of calcium. It is interesting to note here the high concentrations of copper that are favorable to diastatic action, in comparison with the great toxicity of this salt toward plants as observed by KAHLENBERG and TRUE,[24] STEVENS,[25] DUGGAR,[26] LIVINGSTON,[27] and others.

A comparison of the different degrees of the acceleration of diastatic action resulting from the presence of the various salts used singly brings out the point that the effects of potassium chloride and of sodium chloride are much the same; which is in agreement with the work of KUEBEL, GRUTZNER, COLE, WOHLGEMUTH, and others, for extract of pancreas and salivary diastase. Calcium chloride, as shown in the tables, accelerates more than do the salts just mentioned and also more than does magnesium chloride. This is not in accord with the results of WOHLGEMUTH, who, however, used only a single concentration of calcium and magnesium chlorides, this being the optimum concentration for sodium chloride as he found it, a consideration which probably accounts for the discrepancy here mentioned.

EFFECT OF SALT COMBINATIONS

The average ratios of diastatic efficiency furnish a means for attacking the problem which led to the present studies, whether or not two salts simultaneously present in a mixture influence each other's effects on starch hydrolysis. While the data obtained in these experiments are not sufficiently complete to warrant quantitative consideration of this problem, several points are at least qualitatively indicated. It is apparent that, since different concentrations of the same salt produce markedly different effects upon the process of starch hydrolysis, any single treatment may be regarded as a combination of two separate applications of the salt in question, each at a lower concentration. The data above presented show that increasing the concentration of any salt almost never results in a proportionately increased effect upon hydrolysis. Sometimes such an increase completely reverses the direction of the effect, as when an increase in the concentration of sodium chloride from m/128 to m/32 produces an alteration in the efficiency ratio from 0.85 to 1.42. Another example of this is furnished by calcium chloride, for which a change in concentration from m/4 to molecular is accompanied by an alteration of the salt effect on diastatic action, from 33 per cent acceleration to a quantitatively undetermined, but nevertheless exceedingly marked, retardation. In most cases increased concentration of a single salt does not change the direction of the effect, but simply alters its intensity, as when an increase in the concentration of sodium chloride, from m/8 to m/2, results in decreasing the acceleration of hydrolysis from 82 to 71 per cent. It is to be expected, therefore, that the bringing of two different salt treatments into combination may frequently result in the same sort of effect as that produced by increasing the concentration of one of the single salts, the total salt concentration remaining the same as before. This is the true condition of affairs in many instances, and the specific question which confronts us—without attempting any precise quantitative comparisons—is whether combinations of two salts produce more or less effect upon starch hydrolysis than would either salt alone, at the higher concentration that obtains in the combination. Put in another way, the problem is this: if a portion of the amount of a given salt in a diastase mixture be replaced with a molecularly equal amount of another salt, is the resulting alteration in the effect upon hydrolysis merely that traceable to dilution of the first salt together with that due to addition of the second (as would be expected if the combined treatments were without effect upon each other), or is this alteration of a different character? Despite the incompleteness of the data at hand, study of the tables brings out several points bearing upon this question, which are made still more clear if graphs are resorted to. The more striking of these points will now be mentioned.

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[24] KAHLENBERG, LOUIS, and TRUE, RODNEY H., On the toxic action of dissolved salts and their electrolytic dissociation. BOT. GAZ. **22**:81–124. 1896.

[25] STEVENS, F. L., The effect of aqueous solutions upon the germination of fungus spores. BOT. GAZ. **26**:377–406. 1898.

[26] DUGGAR, B. M., Physiological studies with reference to the germination of certain fungus spores. BOT. GAZ. **31**:38–66. 1901.

[27] LIVINGSTON, BURTON EDWARD, Chemical stimulation of a green alga. Bull. Torr. Bot. Club **32**:1–34. 1905.

In the case of the two monovalent salts, with concentrations in the region of m/16 (where the accelerating influence of the single salts is marked, without attaining its maximum), the acceleration of diastatic action produced by combination (molecular proportions 1:1) is notably greater than that produced by either single salt. At the highest concentration used (m), however, the reverse of this proposition holds, and the combination is not as favorable to diastatic action as are the single treatments.

The four series of combinations involving sodium chloride or potassium chloride with the chloride of calcium or magnesium generally show efficiencies which follow somewhat closely those for the bivalent salt of the combination. With combinations of sodium and calcium chlorides (molecular proportions 2:1; equivalent proportions 1:1), in lower concentrations (where the calcium salt alone accelerates more markedly than does that of sodium) the efficiencies, higher than for the sodium salt alone, are not as high for the combination as for calcium chloride alone. In concentrations from 3m/64 to 3m/16 the combination results in greater acceleration than does either single treatment.

Combinations of sodium chloride and magnesium chloride (molecular proportions 4:1, equivalent proportions 2:1) show no cases where the tendency to accelerate hydrolysis is pronouncedly greater than that resulting from one of the single salts. It is best here, for the most rapid hydrolysis, to use magnesium chloride in lower concentrations (where it alone accelerates more) and to use sodium chloride in higher concentrations (where this salt accelerates more).

In concentrations of the region of m/32 the efficiencies for calcium chloride alone are markedly greater than for the combination of this salt with potassium chloride (molecular proportions 2:1; equivalent proportions 1:1), although the partial concentrations of the latter salt, when singly applied, possess a decided accelerating effect. At the concentration 3m/16, diastase efficiency for the combination used is greater than for either salt alone at concentrations nearest 3m/16.

The relations which hold for combinations of potassium chloride and magnesium chloride (molecular proportions 4:1; equivalent proportions 2:1) are similar to those which obtain for combinations of sodium chloride with the magnesium salt. It appears that the combination is here never more favorable for starch hydrolysis than the single salt treatments.

In combinations of magnesium chloride with calcium chloride (molecular proportions 2:1) the efficiencies appear to follow those of the calcium salt, the magnesium in this combination seeming to have acquired as great efficiency for the acceleration of hydrolysis as that which characterizes the portion of the other salt which it replaces.

With combinations of copper chloride and ferric chloride (molecular proportions 1:1) the efficiencies generally lie between those for the single-salt treatments of the same concentration, as though the effectiveness of the combination were the mean influence of the two component partial treatments. No increased efficiency is here brought out by replacing a portion of one of these salts with a molecularly equal portion of the other.

While the considerations just presented appear to lead to the conclusion that certain concentrations of salt combinations may favor greater diastatic activity than do the same concentrations of either salt alone, yet it is obvious that sufficient information is not yet at hand to allow any attempt at generalization. The suggestion becomes patent that the study of properly balanced salt combinations, in their relation to enzymatic action, may add much not only to our knowledge of this persistently vague province of physiology, but also to our ability to control these important processes.

THEORETICAL CONSIDERATION OF RESULTS

In connection with the general problem of the nature of the acceleration and retardation of diastatic action by electrolytes, the results of the investigations of COLE deserve careful consideration. In a research carried on with dialyzed ptyalin, this author formulated a hypothesis to account for the influence of electrolytes on enzyme action, which to quote him directly is as follows (*op. cit.* p. 211): "The hydrolysis of starch by ptyalin is accelerated by the presence in the solution of electronegative ions (anions) other than hydroxyl ions and depressed by the presence of electropositive ions (kations) and by hydroxyl ions." He considers the effect of electrolytes on the rate of action to be compounded of two factors: (1) "the acceleration due to the anion; (2) the depression due to the kation." The work of this author showed that the addition of a small quantity of hydrochloric acid to a chloride in optimum concentration for diastatic hydrolysis did not result in an added acceleration, but in a decreased activity. This he accounted for by considering the acceleration produced by the chloride as due to the chlorine ions, these being already in optimum concentration before the addition of the acid; hence when more chlorine ions were added with the acid there could be no further increase in diastatic activity. The observed decrease in enzyme efficiency, which accompanied the putting in of the acid, was explained as due to the increased concentration of retarding cations which must result from the presence of the acid and to the destruction of ferment by the hydrogen ions. The salt present in a molecular condition is not taken into consideration.

This hypothesis appears to explain many of the phenomena of salt influence upon diastatic action as found in these studies. It does not, however, offer an explanation for the retardation of diastatic action by low concentrations of sodium and potassium chlorides. COLE suggests also that all cations seem to retard diastatic action alike, which would seem questionable from the standpoint of the results obtained in the present investigation.

WOHLGEMUTH, investigating the effects of certain salts on the hydrolysis of starch by pancreatic amylase, using a colorimetric method for determining the rate of hydrolysis, concludes that the acceleration caused by a chloride is due to the chlorine ion. BANG,[28] working with dialyzed ptyalin and determining the amount of a reducing dextrin formed from soluble starch in a given time, as a measure of the rate of hydrolysis, agrees with WOHLGEMUTH in this.

MCGUIGAN, working with malt diastase and using as a criterion for comparing the different salt effects the inhibition of the formation of any reducing sugar in a certain time period, takes a view opposite to that of COLE. He considers the acceleration of enzyme action brought about by a salt as due to the effect of the cation and the retardation as due to the anion. Although his results are not directly comparable to those of the present investigation, it may be noted that most of the salt effects here brought out may be explained by this theory about as satisfactorily as by that of COLE. It is manifestly impossible, however, from the evidence brought out in this investigation, to decide whether either theory is adequate to explain satisfactorily the phenomena of acceleration and retardation of diastatic action by electrolytes. It is, of course, probable that diastatic action is, in some measure, affected by the presence of salts in a molecular condition, also that certain salts form compounds with the enzyme itself, or with other organic bodies which are present in the diastatic mixtures that have usually been used in experimentation of this sort. Likewise diastases of different origins may be influenced quite differently by the same electrolytes. Further and more careful experimentation, with refinement of methods as suggested by FORD,[29] SHERMAN, KENDALL and CLARK, FRANKEL and HAMBURG,[30] and others, are still much needed.

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[28] BANG, IVAR, Untersuchungen über Diastasen. Biochem. Zeitschr. **32**:417–442. 1911.

Summary

The present investigation deals with the effects of sodium, potassium, calcium, magnesium, cupric, and ferric chlorides, alone and in certain binary combinations, on the hydrolytic activity of Merck's "diastase of malt absolute," the enzymatic mixture acting on a boiled solution of washed maize starch, at 50° C. The disappearance of the ability of the starch to give a color reaction with iodine was taken as the end point of the reaction, and the reciprocal of the time period which elapsed before this end point was attained (considering the time period of the control without added salt as unity) was used as a measure of the intensity of enzyme action.

A wide variation is clearly shown in the influence of the different chlorides upon diastatic action, which is probably to be related to the properties of the various cations employed. More or less

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[29] FORD, JOHN S., Lintner's soluble starch and the estimation of diastatic power. Jour. Soc. Chem. Ind. **23**:414–422. 1904.

[30] FRANKEL and HAMBURG, Über Diastase. Beitr. Chem. Physiol. u. Path. **8**:389–398. 1906.

pronounced acceleration of starch hydrolysis is shown for all of the salts used at different concentrations; the highest acceleration found is for iron (291 per cent) and the next highest for calcium (269 per cent). Retardation of hydrolysis is shown at high concentrations for all salts excepting sodium chloride and potassium chloride. For these two salts a pronounced retarding action (15 per cent in both cases) is manifest at low concentrations, the greatest retardation occurring with concentration m/128. This retardation in weak solution seems not to have been considered heretofore.

Combinations of two salts are shown to be sometimes more and sometimes less efficient in modifying diastatic action than are molecularly equal concentrations of their component salts. It is thus possible that enzymatic power, the magnitude of which is frequently to be related to the concentration of single salts in the medium, may in some cases at least be still more highly developed than is possible through the influence of single salts, by the presence of a properly balanced salt combination.

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